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Novel Influenza A Virus (H1N1) RT-PCR Detection Kit Product Insert

Product # 27900

INTENDED USE

Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit is used for the purification and detection of the novel Influenza A virus (H1N1) of swine origin that caused a worldwide pandemic (Phase 6, declared by WHO) in 2009. The kit contains two parts: (1) a novel Influenza A virus (H1N1) RNA purification kit using Norgen's proprietary spin column technology, and (2) a ready-to-use detection kit for rapid and accurate detection of the novel Influenza A virus (H1N1) by end-point RT-PCR.

The kit is designed for the rapid extraction and qualitative detection of the H1 Hemagglutinin viral RNA transcript of the novel Influenza A virus (H1N1) in nasopharyngeal swabs, nasal swabs, throat swabs and nasal aspirates from human patients with signs and symptoms of respiratory infection in conjunction with clinical and epidemiological risk factors. This kit is intended to be used in clinical labs with the ability to perform RT-PCR.

Negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions.

SUMMARY AND EXPLANATION

Influenza is caused by three immunologic types of RNA viruses (A, B and C) within the Orthomyxoviridae family. Seasonal influenza is typically caused by three major subtypes of hemaglutinin (H1, H2 and H3) and two subtypes of neuraminidase (N1 and N2). A novel sub-type of influenza A virus called pandemic H1N1 2009 virus was identified in Mexico and reported by the CDC and WHO in April, 2009 (Novel swine-origin influenza A (H1N1) virus investigation team, 2009; CDC, 2009-a; and Fraser *et al.*, 2009). H1N1 2009 is a novel sub-type virus that transmits easily between humans with 21 countries reporting cases within a month of initial identification (CDC, 2009-b). It is essential that public health laboratories around the world undertake detailed surveillance to monitor the spread and impact of pandemic H1N1 2009 virus as well as try to predict future changes in virulence (Fraser *et al.*, 2009). Methods for the rapid diagnosis, case identification and tracking of this novel pathogen in the human population are therefore required to develop appropriate management strategies to mitigate morbidity and mortality.

H1N1 2009 typically presents with a combination of upper and lower respiratory signs and symptoms, including fever, headache, myalgia and general malaise. Illness severity can range from symptoms which resemble the common cold to severe pneumonia requiring hospitalization. Persons at risk for hospitalization include children under the age of two, adults aged 65 and older and persons with significant co-morbidities. Duration of illness is 2-5 days, but symptoms may last for a week or longer. Novel influenza viruses are first identified by amplification and sequencing of specific genes and these sequences can be compared to those already deposited in databases. Such a methodological approach is important for identification of new viruses but generally requires high viral loads (or cultured virus) for good quality sequence data to be obtained. The turn-around-time required for this type of analysis negates its use as a frontline diagnostic test, especially for large numbers of clinical samples. Since sequences are now available, it is important that public health laboratories develop and validate high throughput sensitive, specific and rapid assays for use in diagnostics and surveillance.

As a rapid response to the outbreak of pandemic H1N1 2009, the Centers for Disease Control (CDC, Atlanta, USA) provided detailed methodology for real-time PCR amplification and detection of this virus (Novel swine-origin influenza A (H1N1) virus investigation team, 2009). Other conventional and real-time RT-PCR protocols have also been published recently for detection of this virus (Poon *et al.*, 2009). Norgen Biotek Corp. has developed a viral RNA extraction and RT-PCR assay for the detection of pandemic H1N1 2009 virus using primers targeting the hemaglutinin (HA) gene from swab samples. This detection kit will aid in the identification of new cases of pandemic H1N1 2009 virus and ensure optimal management to minimize the transmission to vulnerable individuals as well as aid in surveillance activities to understand the full impact of the virus in the community.

PRINCIPLES OF THE PROCEDURES

Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit contains two parts: (1) a novel Influenza A virus (H1N1) RNA purification kit using Norgen's proprietary spin column technology, and (2) a ready-to-use detection kit for rapid and accurate detection of the novel Influenza A virus (H1N1) by end-point RT-PCR. In the first part, the provided RNA purification kit allows the extraction of viral RNA from the various acceptable respiratory specimens. Purification is based on spin column chromatography using Norgen's proprietary resin. The RNA isolated is of the highest quality and free of other cellular components. An Isolation control is provided for spike-in isolation for the subsequent determination of extraction efficiency. In the second part, the purified RNA is subjected to RT-PCR using the provided ready-to-use RT-PCR detection kit. The RNA is directly added to the provided one-step RT-PCR master mix. The reaction involves first the reverse transcription of the viral RNA into cDNA followed by PCR amplification of the H1-specific fragment using a thermo-stable polymerase. An RT-PCR control is provided in all reactions for the determination of RT-PCR efficiency. In addition, a detection control is provided as a positive control for detection. The RT-PCR detection kit is provided with a molecular weight marker for the DNA agarose gel electrophoresis. The detection of the novel Influenza H1N1 virus is determined by the presence of the H1 specific PCR product, and confirmed by the presence of the Isolation Control product as well as the RT-PCR control product.

Kit Components:

Component	Contents
Lysis Solution	30 mL
Wash Solution (concentrate)	11 mL
Elution Buffer	2 mL
Mini Filter Spin Columns	24
Collection Tubes	24
Elution Tubes (1.7 mL)	24
H1N1 2X RT- PCR Master Mix	0.35 mL
H1N1 IsoC**[a]	1.4 mL
H1N1 PosC**[b]	0.05 mL
Nuclease Free-Water	1.25 mL
Norgen's DNA Marker	0.1 mL
Product Insert	1

** IsoC = Isolation Control ; PosC= Positive Control*
[a] The positive control is an in vitro transcribed H1 RNA fragments.
[b] The isolation control is an in vitro transcribed cloned RNA product.

Customer-Supplied Reagents and Equipment

- Benchtop microcentrifuge
- 95-100% ethanol
- Thermocycler and or Real-Time PCR System
- Micropipettes with an accuracy range between 1-10 µL, 10-100 µL and 100-1000 µL
- Laminar flow hood for extractions
- Vortex
- Sterile, nuclease-free aerosol-barrier micropipettor tips
- Microcentrifuge tube rack
- Disposable latex gloves
- β-mercaptoethanol

Storage Conditions and Product Stability

- The Positive Control (***H1N1 PosC,*** blue cap) and Isolation Control (***H1N1 IsoC*****,** orange cap) should be stored at -70°C. If needed, make aliquots of the controls according to the volume used in the protocol (10 µL of ***H1N1 PosC*** or 20 µL of ***H1N1 IsoC***) prior to freezing.
- The 2X RT-PCR Mastermix should be stored at -20°C. Make appropriate aliquots if needed
- All other kit components may be stored at room temperature
- The 2X RT-PCR Mastermix, Postive Control and Isolation Control should not undergo repeated freeze-thaw (a maximum freeze-thaw of three times).
- Do not use kit or reagents past their expiration dates
- Allow reagents to thaw at room temperature prior to use
- After addition of RT-PCR master mix use within one hour
- Kit reagents are stable through the end of the expiration month indicated on the packaging label when stored at the recommended temperatures.

General Precautions

- Follow universal precautions. All patient specimens should be considered as potentially infectious and handled accordingly.
- Diagnostic laboratory work on clinical samples from patients who are suspected of having H1N1 influenza virus infection should be conducted in a BSL2 laboratory. All sample manipulations should be carried out in a biosafety cabinet. Viral isolation on clinical specimens from patients who are suspected of having H1N1 influenza virus infection should be performed in a BSL2 laboratory with BSL3 practices (H1N1 Influenza Virus Biosafety Guidelines for Laboratory Workers: www.cdc.gov/h1n1flu/guidelines_labworkers.htm).
- Wear personal protective equipment, including gloves and lab coats when handling kit reagents. Wash hands thoroughly when finished performing the test.
- Do not smoke, drink or eat in areas where kit reagents and/or human specimens are being used.
- Dispose of unused kit reagents and human specimens according to local, provincial or federal regulations.
- Workflow in the laboratory should proceed in a uni-directional manner, beginning in the pre-amplification area(s) (i.e. specimen collection and RNA extraction) and moving to the amplification / detection area(s) (RT-PCR and gel electrophoresis).
- Do not use supplies and equipment across the dedicated areas of specimen extraction and sample preparation. No cross-movement should be allowed between the different areas.
- Supplies and equipment used for specimen preparation should not be used for pipetting or processing amplified DNA or other sources of target nucleic acids.
- All amplification supplies and equipment should be kept in the amplification / detection area at all times.
- Personal protective equipment, such as laboratory coats and disposable gloves, should be area specific.
- As contamination of patient specimens or reagents can produce erroneous results, it is essential to use aseptic techniques.
- Pipette and handle reagents carefully to avoid mixing of the samples.
- Use proper pipetting techniques and maintain the same pipetting pattern throughout the procedure to ensure optimal and reproducible values.
- Do not substitute or mix reagents from different kit lots or from other manufacturers.
- Do not interchange reagent tube / bottle caps as this may lead to contamination and compromise test results.
- Only use the protocol provided in this insert. Alterations to the protocol and deviations from the times and temperatures specified may lead to erroneous results.

Quality Control

In accordance with Norgen's ISO 9001 and ISO 13485-certified Quality Management System, each lot of Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit, including the H1N1 2x RT-PCR Master Mix, H1N1 Isolation Control and H1N1 Positive Control are tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations
Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit is designed for research purposes only. It is not intended for human or diagnostic use.

Product Warranty and Satisfaction Guarantee
NORGEN BIOTEK CORPORATION guarantees the performance of all products in the manner described in our product manual. The customer must determine the suitability of the product for its particular use.

Safety Information
Biosafety level 2 practices are recommended for works involving clinical samples from patients who are suspected of having H1N1 influenza virus infection. Ensure the appropriate containment equipment and facilities are used for activities involving cultures or potentially infectious clinical materials. Ensure that a suitable lab coat, disposable gloves and protective goggles are worn when working with chemicals. For more information, please consult the appropriate Material Safety Data Sheets (MSDSs). These are available as convenient PDF files online at *www.norgenbiotek.com*.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

The **Lysis Solution** contains guanidine salts, and should be handled with care. Guanidine salts form highly reactive compounds when combined with bleach, thus care must be taken to properly dispose of any of these solutions.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

INSTRUCTIONS FOR USE

Notes Prior to Use

- All centrifugation steps are carried out in a benchtop microcentrifuge at 14,000 x g (~ 14,000 RPM) except where noted. All centrifugation steps are performed at room temperature.
- A variable speed centrifuge should be used for maximum kit performance. If a variable speed centrifuge is not available a fixed speed centrifuge can be used, however reduced yields may be observed.
- Ensure that all solutions are at room temperature prior to use.
- Prepare a working concentration of the **Wash Solution** by adding 25 mL of 95% ethanol (provided by the user) to the supplied bottle containing the concentrated **Wash Solution**. This will give a final volume of 36 mL. The label on the bottle has a box that may be checked to indicate that the ethanol has been added.
- **Add 10 µL of β-mercaptoethanol** (provided by the user) to each 1 mL of Lysis Solution required. β-mercaptoethanol is toxic and should be dispensed in a fume hood.
- It is important to work quickly during this procedure.

A. SPECIMEN COLLECTION AND LYSATE PREPARATION

Acceptable specimen types include nasal swabs, nasopharyngeal swabs, throat swabs or nasal aspirates in viral transport medium tube or equivalent. If using swabs, use only sterile Dacron, nylon or rayon swabs with plastic shafts. Note: Do not use calcium alginate swabs as they may contain substances that are inhibitory to PCR.

This kit is also compatible with samples collected using Norgen's Sample Collection Kit For Upper Respiratory Tract Infectious Agents (Cat #29100). Please follow the instructions provided with that kit for specimen collection and preservation.

i. Specimens previously collected using Norgen's Sample Collection Kit For Upper Respiratory Tract Infectious Agents (Cat #29100)

1) Transfer 300 µL of preserved specimen to an RNase-free microcentrifuge tube.
2) Add 300 µL of the **Lysis Solution** and vortex for 10 seconds to mix.
3) Add 20 µL of the Isolation Control (***H1N1 IsoC***) to the lysate. Vortex for 10 seconds to mix.
4) Add 300 µL of 95% ethanol to the lysate. Vortex for 10 seconds to mix.
5) Proceed to RNA Isolation (Step B).

ii. Specimen collection from swab in media:

1) If swab is transported in media the user should transfer 200 µL to an RNase-free microcentrifuge tube.
2) Add 500 µL of the **Lysis Solution** and vortex for 10 seconds to mix.
3) Add 20 µL of the Isolation Control (***H1N1 IsoC***) to the lysate. Vortex for 10 seconds to mix.
4) Add 400 µL of 95% ethanol to the lysate. Vortex for 10 seconds to mix.
5) Proceed to RNA Isolation (Step B).

iii. Specimen collection directly from swab:

1) Alternatively, dry swabs can be placed directly into an RNase-free microcentrifuge tube containing 1 mL of the **Lysis Solution**.
2) Using sterile techniques, cut the tip where the nasal or throat cells were collected and place into microcentrifuge tube containing the **Lysis Solution**.
3) Close the tube and vortex for 1 minute to release the virus particles.
4) Using a sterile pipette transfer, 400 µL of the lysate into another RNase-free microcentrifuge tube.
5) The extraction control (EC) (20 µL) should then be added to the Lysis tube and the tube vortexed for 10 seconds to mix.
6) Add 200 µL of 95% ethanol to the lysate. Vortex for 10 seconds to mix.
7) Proceed to RNA Isolation (Step B).

B. SPECIMEN RNA PURIFICATION

Following the lysate preparation viral RNA can be extracted from the patient specimens using the supplied buffers and solutions according to the following protocol:

1. Assemble a column with one of the provided collection tubes.
2. Apply the lysate with ethanol (up to 650 µL) to the column and centrifuge for 1 minute at 14,000 rpm.

   Note: Ensure the entire lysate volume has passed through into the collection tube by inspecting the column. If the entire lysate volume has not passed through, spin for an additional minute.

3. Discard the flowthrough and reassemble the spin column with its collection tube.
4. Depending on lysate volume, repeat steps **B2** and **B3**.
5. Apply 400 µL of Wash solution and centrifuge for one minute at 14,000 rpm.

   Note: Ensure the entire wash solution has passed through into the collection tube by inspecting the column. If the entire wash volume has not passed through, spin for an additional minute.

6. Discard the flowthrough and reassemble the spin column with its collection tube.
7. Repeat steps **B5** and **B6** two more times.
8. Spin the column for 2 minutes to thoroughly dry the resin at 14,000 rpm. Discard the collection tube.
9. Place the column into a new 1.7 mL Elution tube.
10. Add 50 µL of Elution Solution to the column.
11. Centrifuge for 2 minutes at 2,000 rpm followed by a 2 minute spin at 14,000 rpm. Note the volume eluted from the column. If the entire 50 µL has not been eluted, spin the column for an additional minute at 14,000 rpm.

12. The purified RNA sample could be used immediately for RT-PCR as described below. It is recommended that samples be placed at -70°C for long term storage.

C. H1N1 RT-PCR Assay Preparation

Notes:

- Before use, suitable amounts of all RT-PCR components should be completely thawed at room temperature, vortexed and centrifuged briefly.
- The amount of H1N1 2X RT-PCR Master Mix provided is enough for up to 32 RT-PCR reactions (24 sample PCR, 4 positive control PCR and 4 no template control PCR).
- For every PCR run, one reaction containing H1N1 Positive Control (***H1N1 PosC***) and one reaction as no template control must be included for proper interpretation of results.
- The recommended minimum number of RNA samples tested per PCR run is 6.

1. Prepare the RT-PCR for sample detection as shown in Table 1 below. The recommended amount of sample RNA to be used is 5 µL. However, a volume between 1 and 10 µL of sample RNA may be used as template. Adjust the final volume of the RT-PCR reaction to 20 µL using the Nuclease-Free Water provided.

Table 1. RT-PCR Assay Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
***H1N1* 2X RT-PCR Master Mix**	**10 µL**
Sample RNA	**1 to 10 µL**
Nuclease-Free Water	**Up to 10 µL**
Total Volume	**20 µL**

2. For every RT-PCR run, prepare **one** positive control RT-PCR as shown in Table 2 below:

Table 2. RT-PCR Positive Control Preparation

RT-PCR Components	Volume Per RT-PCR Reaction
***H1N1* 2X RT-PCR Master Mix**	**10 µL**
H1N1 PosC	**10 µL**
Total Volume	**20 µL**

3. For every RT-PCR run, prepare **one** no template control RT-PCR as shown in Table 3 below:

Table 3. RT-PCR Negative Control Preparation

PCR Components	Volume Per PCR Reaction
***H1N1* 2X RT-PCR Master Mix**	**10 µL**
Nuclease-Free Water	**10 µL**
Total Volume	**20 µL**

D. H1N1 RT-PCR Assay Programming

1. Program the thermocycler according to the program shown in Table 4 below.
2. Run RT-PCR.

Table 4. H1N1 Assay Program

PCR Cycle	Step	Temperature	Duration
Cycle 1	**Step 1**	**50°C**	**25 min**
Cycle 2	**Step 1**	**95°C**	**5 min**
Cycle 3 (40x)	**Step 1**	**94°C**	**15 sec**
	Step 2	**64°C**	**30 sec**
	Step 3	**72°C**	**45 sec**
Cycle 4	**Step 1**	**72°C**	**5 min**
Cycle 5	**Step 1**	**4°C**	**∞**

E. H1N1 RT-PCR Assay Results Interpretation

1. For the analysis of the PCR data, the entire 20 µL PCR reaction should be loaded on a 1X TAE 2 % Agarose DNA gel along with 10 µL of Norgen's DNA Marker (provided).
2. The PCR products should be resolved on the 1X TAE 2 % Agarose gel at 150V for 30 minutes.
3. Sample results are provided below:

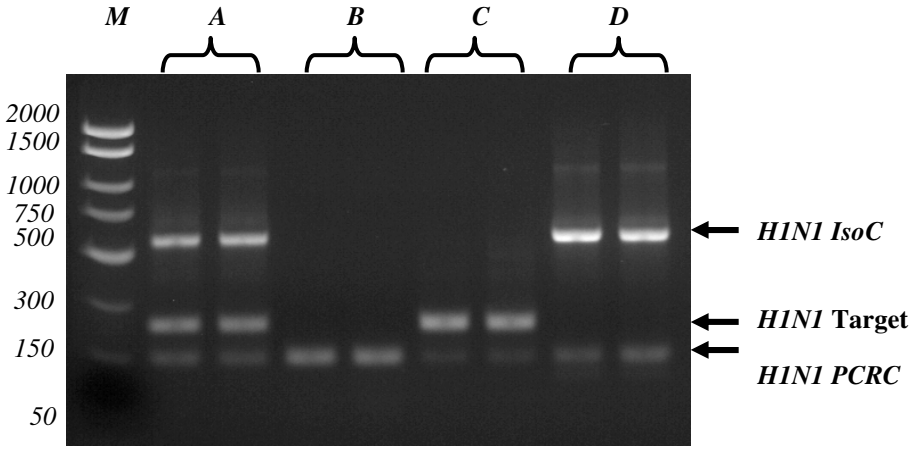

Figure 1: A representative 1X TAE 2% agarose gel showing the amplification of *H1N1* under different conditions (*H1N1* Target). The size of the *H1N1* target amplicon corresponds to 222bp as represented by the provided DNA Marker (M). The size of the *H1N1* Isolation Control (*H1N1 IsoC*) corresponds to 499bp as represented by the provided DNA Marker (M). The *H1N1* 2X RT-PCR Master Mix contains an *H1N1* RT-PCR Control (*H1N1 PCRC*). The *H1N1* PCRC Controls for PCR inhibition. The size of the *H1N1* PCRC corresponds to 142bp as represented by the provided DNA Marker (*M*). The amplification from each lane is interpreted as follows:

Lane A: **Positive Control** or ***H1N1* Detected** – All three RT-PCR amplicons were detected
Lane B: No Template Control – Only ***H1N1 PCRC*** was detected
Lane C: *H1N1* Detected – Lane C showed detection of both the ***H1N1* Target** and ***H1N1 PCRC.*** The result is considered positive detection of *H1N1.*
Lane D: *H1N1* Not Detected – Detection of ***H1N1 IsoC*** and ***H1N1 PCRC***, suggesting that the RNA isolation was successful but no *H1N1* RNA was present in the sample

Table 5. Interpretation of RT-PCR Assay Results

Input Type	*H1N1* IsoC Band (499 bp)	*H1N1* Target Band (222 bp)	*H1N1* PCRC Band (142 bp)	Interpretation
Positive Control	X	X	X	Valid
Negative Control			X	Valid
Sample	X	X	X	Positive
Sample	X		X	Negative
Sample		X	X	Positive
Sample	X	X		Positive
Sample		X		Positive

** For results obtained that are not covered in Table 5 above, please refer to the Troubleshooting Section.

F. H1N1 RT-PCR Assay Specificity

- The specificity of Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit is first and foremost ensured by the selection of the H1-specific primers, as well as the selection of stringent reaction conditions. The primers were checked for possible homologies to all in GenBank published sequences by sequence comparison analysis. The specific detectability of all relevant strains has thus been ensured by a database alignment and by PCR amplification with the following commonly found seasonal flu viruses:
    - H1N1
    - H3N2
    - Avian H5N1
    - Influenza B

G. H1N1 RT-PCR Assay Specificity

- The linear range (analytical measurement) of Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit was determined by analysing a dilution series of a *H1N1* quantification standard ranging from 1×10^{7} copies/µl to 1×10^{-1} copies/µl.
- Each dilution has been tested in replicates (n = 4) using Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit on 1X TAE 2% Agarose gel.
- The linear range of Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit has been determined to cover concentrations from 5×10^{4} copies/mL to at least 1×10^{9} copies/mL

Frequently Asked Questions

1. How many samples should be included per RT-PCR run?

- Norgen's Novel Influenza A Virus (H1N1) RT-PCR Detection Kit is designed to test 24 samples. For every 6 samples, a non-template control and a Positive Control must be included. It is preferable to pool and test 6 samples at a time.

2. How can I interpret my results if neither the *H1N1* PCR control nor the *H1N1* Isolation Control amplifies?

- If neither the *H1N1* PCR control nor the *H1N1* Isolation Control amplifies, the sample must be re-tested. If the positive control showed amplification, then the problem occurred during the isolation, where as if the Positive control did not amplify, therefore the Problem has occurred during the setup of the PCR assay reaction.

3. How should it be interpreted if only the *H1N1* PCR control showed amplification but neither the *H1N1* target nor the *H1N1* Isolation control amplified for a sample?

- This indicates a poor isolation. The isolation procedure must be repeated.

4. How should it be interpreted if only the *H1N1* Isolation Control was amplified in a sample?

- The sample tested can be considered as *H1N1* negative.

5. How should it be interpreted if only the *H1N1* target and the *H1N1* PCR control were amplified in a sample?

- The sample tested can be considered as *H1N1* positive.

6. How should it be interpreted if only the *H1N1* target was amplified in a sample?

- The sample tested should be considered as *H1N1* positive. At high *H1N1* copies input, the *H1N1* amplicon will be predominant and thus the *H1N1* PCR control as well as the *H1N1* Isolation control may not amplify as they compete for PCR resources.

7. How should it be interpreted if only the *H1N1* PCR control and the *H1N1* Isolation control showed amplification in a sample?

- The sample tested can be considered negative

8. What If I forgot to do a dry spin after my second wash?

- Your RNA elution will be contaminated with the Wash Solution. This may dilute the RNA yield in your elution and it may interfere with the RT-PCR detection, as ethanol is known to be a PCR inhibitor.

9. What If I forgot to add the *H1N1* Isolation control during the Isolation?

- It is recommended that the isolation is repeated.

Reference

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Technical Assistance

NORGEN's Technical Service Department is staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of NORGEN products. If you have any questions or experience any difficulties regarding Norgen's Urine DNA Isolation Mini Kit (Slurry Format) or NORGEN products in general, please do not hesitate to contact us.

NORGEN customers are a valuable source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at NORGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please contact our Technical Support Team between the hours of 8:30 and 5:30 (Eastern Standard Time) at (905) 227-8848 or Toll Free at 1-866-667-4362. or call one of the NORGEN local distributors (www.norgenbiotek.com) or through email at techsupport@norgenbiotek.com.

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